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Report

Males Use Multiple, Redundant Cues to Detect Mating Rivals

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Summary

Across many species, males exhibit plastic responses when they encounter mating rivals [1]. The ability to tailor responses to the presence of rivals allows males to increase investment in reproduction only when necessary. This is important given that reproduction imposes costs [2] that limit male reproductive capacity, particularly when sperm competition occurs [3, 4]. Fruitfly (*Drosophila melanogaster*) males exposed to rivals subsequently mate for longer and thus accrue fitness benefits under increased competition [5], in line with theory [6, 7]. Here, we show that male *D. melanogaster* detect rivals by using a suite of cues and that the resulting responses lead directly to significant fitness benefits. We used multiple techniques to systematically remove auditory, olfactory, tactile, and visual cues, first singly and then in all possible combinations. No single cue alone was sufficient to allow males to detect rivals. However, the perception of any two cues from sound, smell, or touch permitted males to detect and respond adaptively to rivals through increased offspring production. Vision was only of marginal importance in this context. The findings indicate adaptive redundancy through the use of multiple, but interchangeable, cues. We reveal the robust mechanisms by which males assess their socio-sexual environment to precisely attune responses via the expression of plastic behavior.

Results

In order to respond appropriately to the presence of rivals, males must accurately sample their socio-sexual environment to assess the level of competition they are likely to face. Roles for single cues (i.e., smell and song) in rival detection have been reported in several species [8–11], but the ultimate fitness effects of responding to those cues is not yet known, nor are the effects of responses initiated by the detection of multiple cues.

We tackled this significant gap in our knowledge through a systematic investigation of the potential cues used by male *D. melanogaster* to detect rivals and initiate responses that lead directly to substantial fitness benefits. Previously, we showed that male *D. melanogaster* that are exposed to rivals prior to mating subsequently mate for longer than controls [5] and have significantly higher fitness [5], associated with increased transfer of seminal-fluid proteins [12]. The responses of males to rivals are precisely calibrated on the basis of the length of exposure to other males prior to mating and are only initiated after at least 24 hr of exposure to rivals [13]. The key outstanding question is how males detect the presence of rivals and calibrate their responses according to the prevailing risk of sperm competition. We tested this here by identifying the cues that males use to detect and respond adaptively to the presence of rivals.

We systematically removed the potential cues of sound, smell, touch, and vision by manipulating the focal male's ability to receive, or a rival's ability to produce, these signals. For each cue-removal manipulation, we compared the mating duration of manipulated males that were either exposed or not exposed to a rival for 3 days prior to mating. Crucially, this procedure provided internal controls for each independent test that employed loss-of-function mutants or other manipulations. In most experiments, we also fully controlled for the genetic background by using the wild-type.

We first used multiple techniques to investigate the effect of single cues, namely sound, smell, touch, and vision, on a male's ability to detect rivals. To remove auditory cues we (1) used two deaf mutants to remove a focal male's ability to hear (*inactive* mutation [14]), (2) removed the production of courtship song by rivals by using *vestigial*^{1} males (whose wings are greatly reduced and incapable of producing normal song), or (3) used wild-type rivals whose wings were removed. To test olfactory cues, we removed a male's sense of smell by surgically removing the third segment of the antennae, which contains sensillae bearing the odorant receptors required for males to respond to the odors of other flies [15]. Antennal removal also removes the aristae, which detect sound. Hence, to test olfactory cues alone we also used mutants lacking *odorant receptor 83b* (*Or83b*), a co-receptor necessary for odorant perception in toto [16]. To replicate potential olfactory cues in the absence of a rival, we exposed males to whole male cuticular hydrocarbon (CHC) extracts or to synthetic cis-vaccenyl acetate (cVA). cVA is a male-specific volatile pheromone associated with aggregation [17], aggression [18], and courtship [19] and was an obvious target in the search for cues used in rival detection. To remove tactile cues, we separated males from rivals by using porous netting. This manipulation could also potentially remove gustatory cues by preventing males from "tasting" each other through direct contact. To distinguish these alternatives, we conducted an experiment in which we provided gustatory (and olfactory) cues by coating the internal surface of vials with male CHCs. Finally, to remove visual cues we used either wild-type males held in darkness or vision-defective [20] *white* flies (w^{1118} backcrossed into the wild-type background) held under normal light conditions.

Sets of tests were performed simultaneously on different days, on each of which we also performed an unmanipulated wild-type control experiment. However, every manipulation had its own internal control (in which flies were exposed to rivals or not) and was therefore an independent test. We adopted this procedure to control for the random, unexplained, and often significant variation in *Drosophila* mating behavior across days [21]. Corrections for multiple comparisons were therefore not appropriate. Nevertheless, we applied caution in interpreting results of marginal significance.

Our multiple independent tests revealed that the male response to rivals under unmanipulated control conditions

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was remarkably consistent, with a ~25% increase in mating duration for males exposed to a rival. However, both the absolute value and magnitude of the difference in mating duration in unmanipulated controls varied significantly across days (Supplemental Information). This supports the use of internal controls for each treatment and shows that each test should be considered independently.

Removal of single cues by multiple means had no significant effect on a male's response to rivals (Table 1 and Figures S1 and S2). In addition, males did not increase mating duration in response to the presence of either the full suite of male CHCs or synthetic cVA (Figures S3A and S3C), even though cVA was detectable by males in the experimental paradigm used (Figure S3D). The antenna-removed (olfaction-defective) flies were also auditory defective [22]. However, manipulation of olfactory cues alone via different methods (use of smell mutants; application of cVA and male CHCs) gave identical results (Figures S2 and S3), showing that the ability to smell is not, on its own, sufficient to allow males to detect rivals. Overall, the data therefore reveal that males do not use any single cue of sight, sound, touch, or smell in isolation to respond adaptively to rivals.

We then ran further independent tests by using a fully wild-type genetic background to systematically remove cues in pairs and triplets and then all four in combination (see methods in Supplemental Information). This showed that eliminating any two cues involving sound, smell, or touch fully abolished male responses to rivals (Table 1 and Figure 1). The multiple cues that males used to detect rivals were entirely interchangeable: any paired combination of auditory, olfactory, or tactile cues had the same effect of abolishing male responses to rivals (Table 1). Vision was of minor importance in detecting rivals (it was marginally nonsignificant in combination with olfaction and marginally significant in combination with touch; Table 1). The use of nets to separate males from rivals could block tactile or gustatory cues. Hence, we also ran tests to determine the relevant cue that males used in combination with smell to respond adaptively to rivals. Single males provided with gustatory and olfactory cues (these males were housed in vials whose internal surface was painted with male CHC extract) did not respond by extending mating duration (Figure S3C). This indicates that touch is the important cue to which males respond in combination with either smell or sound and that taste itself is not used as a cue to detect rivals in this context. We conclude that males primarily use any two of the three cues of sound, smell, and touch to detect and respond to rival males.

The results also reveal information about the way in which auditory cues are perceived. *Or83b*^{2} males that lack an arista (i.e., that cannot perceive olfactory or auditory cues [22]) did respond to rivals (Figures S2A and S2B). However, responses to rivals were abolished in *Or83b*^{2} males exposed to rivals that could not sing (Figure S2D). These results show that the song of a rival male is the important component of auditory cues and that this auditory cue is perceived through a route that bypasses the aristae.

Finally, we tested whether the ability to perceive rivals resulted in the fitness effects we documented previously [5]. We repeated the removal of auditory and tactile cues both singly and in combination and counted the total offspring produced in the 24 hr after mating. In line with our previous work [5], in control and single-cue-removed experiments, males exposed to rivals mated for significantly longer than their counterparts held alone (Figure 2A, control $z = 4.141$, n = 76, $p < 0.0001$; auditory removal (−A), $z = 2.662$, n = 73, p = 0.008; tactile removal (−T), $z = 2.060$, n = 66, p = 0.039) and fathered significantly more offspring (Figure 2B, control $t = 2.380$, df = 70, p = 0.020; −A $z = 3.137$, n = 70, p = 0.002; −T $z = 2.068$, n = 66, p = 0.039). When both auditory and tactile cues were removed (−AT), there was no response by males to rivals, i.e., no significant difference in mating duration (Figure 2A, $z = 1.614$, n = 73, p = 0.106) and, as therefore expected, no significant difference in the number of offspring produced (Figure 2B, $z = 1.623$, n = 71, p = 0.105).

Discussion

We found that males use multiple cues to detect rivals and respond adaptively to them. Combinations of auditory, olfactory, and tactile cues were essential for males to detect rivals, and any combination of two of these three cues resulted in equivalent responses. Vision, however, was of only marginal importance as a cue in combination with either smell or touch. The results indicate the existence of adaptive redundancy, that is, the use of multiple, but interchangeable, cues in the detection of male rivals.

To date, scent [8–10] and song [11] have been identified as single cues used for assessment of rivals in several species; however, those studies did not measure the fitness consequences of rival detection. Meadow vole (*Microtus pennsylvanicus*) males that mate in the presence of odors from other males transfer more sperm to females [8], male *Tenebrio molitor* beetles increase the duration of copula and length of post-copula association with the female after exposure to odors mimicking high male density [9], and male newts (*Lissotriton boscai*) alter their courtship behavior after exposure to the chemical cues from other males [10]. In addition, the acoustic environment encountered by juvenile *Teleogryllus oceanicus* field cricket males alters their subsequent mating behavior, investment in reproductive tissue, and condition [11]. Touch has not previously been identified as a cue for responding to rivals. However, touch is a stimulus for the switch from solitary to gregarious behavior in locusts (*Schistocerca gregaria*) [23] and also increases developmental rate in gregarious cockroaches (*Blattella germanica*) [24]. In two separate tests in our study, males retained the ability to respond to rivals after removal of tactile cues alone (Table 1 and Figure 2). These effects were significant and repeatable but relatively subtle (p = 0.038 and p = 0.039, respectively). However, responses to rivals were also fully abolished after removal of auditory and olfactory cues (i.e., in the presence of full tactile cues) (Table 1 and Supplemental Information). Hence, together these findings confirm that touch by itself is not a major, or even essential, modality used in detecting rivals. It can, however, be important when used in combination with other cues (Table 1). In combination with either touch or smell, vision was of only marginal significance in the detection of rivals. In contrast, visual signals play an important role in male-male competition [25], for example in assessment of rivals prior to physical contests [26]. The potential differences in the relative importance of vision in the detection of rivals versus detection of mates might have some interesting implications for the hard wiring of behavior, and they deserve further study. Overall, our study demonstrates the importance of considering all potential cues, singly and in combination, in order to fully identify those of importance to fitness.

Our results identify the importance of chemical, tactile, and auditory cues and also reveal the precise nature of the mechanisms likely to be at work. For example, the tactile cues used

Table 1. Summary of the Effect of Cue Removal on a Male's Ability to Respond Adaptively to the Presence of Rivals

Cue(s) Removed				Male Responses to Rivals: Comparison between Males Kept with or without Rivals prior to Mating			Manipulations Employed	UC
♪				n = 54	$z = 3.567$	$p < 0.0001$	*inactive*[1] (hearing defective) #1	*
♪				n = 65	$z = 2.539$	$p = 0.011$	*inactive*^{3621} (hearing defective) # 2	*
♪				n = 69	$z = 2.907$	$p = 0.004$	*vestigial*[1] (wing-defective) rival	*
♪				n = 76	$z = 3.845$	$p < 0.0001$	wild-type wingless rival	*
	∿			n = 59	$z = 3.845$	$p = 0.002$	antenna-removal experiment #1	*
	∿			n = 66	$z = 2.172$	$p = 0.030$	antenna-removal experiment #2	***
	∿			n = 68	$z = 3.406$	$p = 0.001$	*Or83b*[2] (odorant coreceptor) mutant	**
		✋		n = 71	$z = 2.071$	$p = 0.038$	netted vial	**
			👁	n = 74	$z = 2.859$	$p = 0.004$	wild-type held in darkness	ns
			👁	n = 69	$z = 3.297$	$p = 0.001$	w*hite* (vision defective)	ns
♪			👁	n = 51	$z = 3.398$	$p = 0.001$	wingless rival, w*hite* (vision defective)	**
		✋	👁	n = 51	$z = 2.070$	$p = 0.038$	netted vial, w*hite* (vision defective)	**
	∿		👁	df = 32	$t = 1.990$	$p = 0.055$	antenna removed, w*hite* (vision defective)	**
♪		✋		n = 73	$z = 1.389$	$p = 0.165$	wingless rival, netted vial	**
♪	∿			df = 55	$t = 0.333$	$p = 0.740$	wingless rival, antenna removed	***
	∿	✋		n = 67	$z = 0.391$	$p = 0.696$	antenna removed, netted vial	***
♪	∿	✋		n = 73	$z = 1.584$	$p = 0.113$	wingless rival, antenna removed, netted vial	*
♪	∿		👁	n = 49	$z = 0.313$	$p = 0.755$	wingless rival, antenna removed, w*hite* (vision defective)	*
♪		✋	👁	n = 48	$z = 0.518$	$p = 0.604$	wingless rival, netted vial, w*hite* (vision defective)	**
	∿	✋	👁	df = 45	$t = 1.778$	$p = 0.080$	antenna removed, netted vial, w*hite* (vision defective)	*
♪	∿	✋	👁	df = 38	$t = 0.914$	$p = 0.360$	wingless rival, antenna removed netted vial, w*hite* (vision defective)	***

♪ auditory, ∿ olfactory, ✋ tactile, and 👁 visual cues are indicated. Males were scored as responding to rivals if there was a significant difference within each cue-removal treatment between the mating duration of males kept with and without a rival in the 3 days prior to mating. Results of either Student's t tests or Mann-Whitney tests are given (see main text and Supplemental Information for further details). Shading incicates nonsignificant differences, i.e., when responses to rivals are abolished. For each set of experiments run simultaneously, an unmanipulated control (UC) treatment was included (single wild-type males versus pairs). The significance of the UC result for each set of tests is shown (ns = non significant, * $p < 0.05$, ** $p < 0.01$,*** and $p < 0.0001$). See also Figure S1.

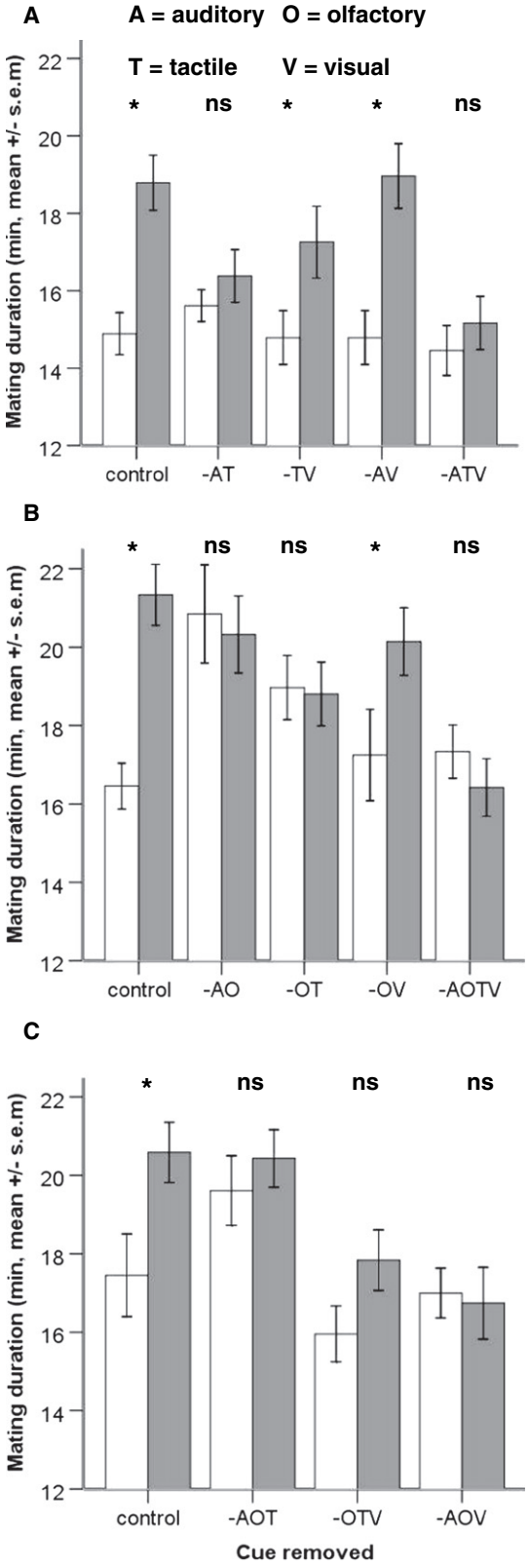

Figure 1. Effect of Cue Removal on the Responses of Males to Their Rivals

A male's response to rivals was measured as the difference in mating duration between males either not exposed (white) or exposed (gray) to a rival prior to mating. Three sets of experiments (A, B, and C) are shown. The tests were an unmanipulated wild-type control, or removal of combinations of auditory (−A), olfactory (−O), tactile (−T), or visual (−V) cues. (*, significant difference; ns, nonsignificant; see Table 1 for exact p values). Removal of any combination of two cues out of A, O, or T eliminated the ability of males to respond to rivals. See also Figure S2.

by males are separate from the effects of any olfaction sensed through direct contact; the effects of touch and smell could be separated, and both were needed for males to respond to rivals. In addition, when provided with olfactory and gustatory cues, single males did not respond as if exposed to a rival, whereas ablation of olfactory and tactile cues (via the use of nets to separate males) did abolish male responses to rivals. This identifies touch rather than gustation as a cue used in rival detection. Similarly, the results suggest that the detection of courtship song was the essential component of auditory cues: the detection of song, rather than hearing per se was (in combination with other cues) required for males to respond to rivals. Intriguingly, the experiments employing removal of the aristae (which removes hearing [22]) show that the perception of song occurs through a route that bypasses the aristae. Because hearing and mechanosensation have genetically similar transduction mechanisms [22], it is possible that vibrations produced during song are detected instead by vibrosensory chordonatal organs [22]. In addition, wing vibrations produced during song might help to disperse CHCs and thus increase olfactory stimulation. These possibilities underscore the central finding that multiple cues are used in the detection of rivals.

The finding that multiple, but interchangeable, cues are essential for adaptive responses by males is an intriguing result. The significance of the use of redundant versus fixed cues is not clear. Although relevant theory remains to be developed, a useful starting point is the models that seek to explain the evolution and maintenance of multiple signaling under female mate choice. In this context, it has been suggested that multiple signals convey different information, are additive [27, 28], or are aimed at multiple receivers, such as male and female conspecifics [29]. Signals could be directed toward both mates and rivals, leading to the evolution of complex signaling traits [30]. Environments that fluctuate rapidly (such as the social milieu) could drive the evolution of multiple signals [31]. However, in most scenarios considered to date, the signaler is selected to convey a message to the receiver. In our experiments there is a benefit to the receiver (the male that detects rivals and obtains matings) but not to the signaler (rival males that are detected but that do not mate). This situation is reminiscent of sexual-conflict models in which females are selected to use information from multiple traits if male signals are not honest indicators of quality [32]. Our results highlight an exciting opportunity to pursue novel theory in the area of the multi-component signaling cues used by males to respond adaptively to rivals.

We suggest that in order to precisely calibrate their responses to rivals, males require information that the individuals in the immediate proximity are of the same species, are male, and are likely to be of competitive importance. The different cues we have identified as signaling the presence of rivals might transmit these multiple strands of information. For example, song and smell might convey species and sex identification [33, 34], and touch might indicate the proximity and persistence of competitors. However, this does not explain why all three cues are not needed, nor why cues are fully interchangeable. Alternatively, all cues might feed into one reaction pathway containing a threshold that must be

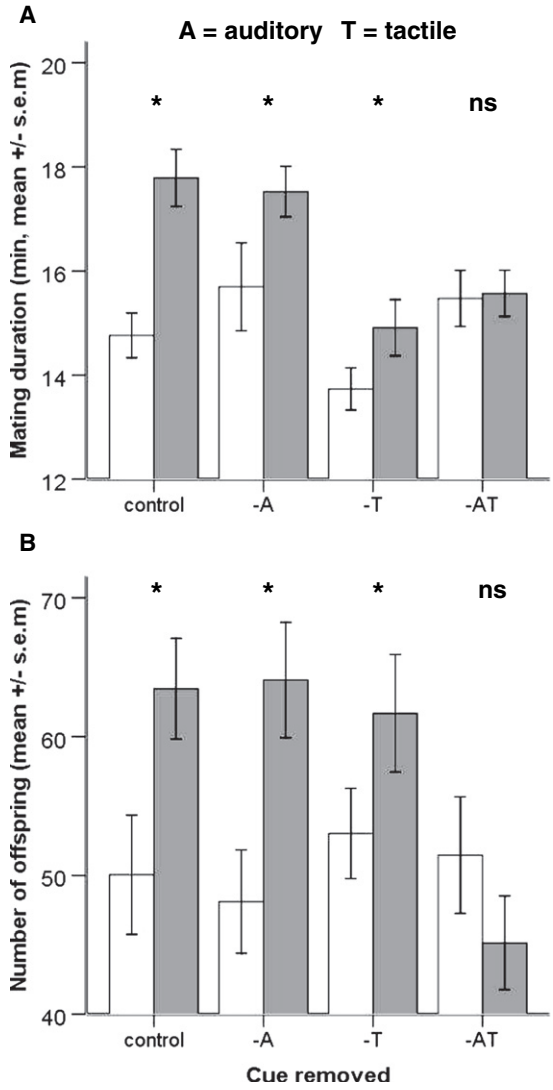

Figure 2. Male Responses to Rivals Significantly Increase Mating Duration and Number of Offspring Fathered

Adaptive responses by males to their rivals: (A) mating duration and (B) number of offspring produced in 24 hr after mating. Males were either not exposed (white) or exposed (gray) to a rival prior to mating. The tests were an unmanipulated wild-type control, or removal of auditory (−A) and/or tactile (−T) cues. (*, significant difference in mating duration; ns, nonsignificant; see main text for exact p values). Significant extensions to mating duration led directly to the production of significantly more offspring (in control, −A, and −T treatments). In contrast, when mating duration did not differ, neither did offspring production (−AT treatment). See also Figure S3.

exceeded in order for a male to respond to rivals. Indeed, our previous work suggests that the competitive importance of rivals is conveyed by a minimum length of exposure to them [5, 13]. Moreover, the consistency of the male response, and its initiation only through perception of signals from multiple sources, demonstrates the importance to fitness of male short-term responses to socio-sexual situations. Hence, to avoid off-target, costly reproductive investment, males might use multiple cues as a robust and reliable source of information. The multi-component signaling system we have revealed here might also enable males to avoid "evolutionary traps." These can occur if male reproductive behavior is either misdirected [35] (e.g., when "matings" occur between male buprestid beetles and beer bottles [36]) or exploited [35]. Such examples serve to show the importance of honest cues that prevent males from potentially costly mistakes.

Our results demonstrate that investigations of signals in isolation are potentially misleading and that the evolution of multiple sexual signaling needs to be considered in a wider context. Overall, the results reveal the depth and complexity with which males can sample their reproductive environment and the robustness with which they can respond adaptively to their sexual surroundings through the expression of plastic behavior.

Supplemental Information

Supplemental information includes supplemental experimental procedures, supplemental results, and three figures and can be found with this article online at doi:10.1016/j.cub.2011.03.008.

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